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Polyphasic characterization of *Aspergillus* section *Flavi* isolated from animal feeds in Algeria

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Abstract

In Algeria, little information is available on the population structure of *Aspergillus* section *Flavi* in raw materials and resultant animal feeds. A total of 172 isolates belonging to *Aspergillus* section *Flavi* were recovered from 57 animal feeds and identified on the basis of macro and micro-morphological characters, mycotoxin production and genetic relatedness. For the molecular analysis, sequencing of the calmodulin gene (CaM) and the Internal Transcribed Spacer (ITS) regions were performed for representative isolates. Four distinct morphotypes were distinguished: *A. flavus* (78.5%), *A. tamarii* (19.2%), *A. parasiticus* (1.7%) and *A. alliaceus* (0.6%). All *A. flavus* isolates were of the L type and no correlation between sclerotia production and aflatoxigenicity was observed. Our results showed that 68% of the *A. flavus* strains produced aflatoxins B (AFB), and 72.7% were cyclopiazonic acid (CPA) producers. The three isolates of *A. parasiticus* were able to produce AFB and aflatoxins G but not CPA whereas, all the strains of *A. tamarii* produced only CPA. The obtained results revealed the

presence of different species of *Aspergillus* section *Flavi*, amongst which were aflatoxin producers. This study provides evidence useful for considerations in aflatoxin control strategies.

Practical applications

This is the first report about aflatoxins production by *Aspergillus* section *Flavi* species in both raw materials (maize, soybean, wheat bran and barley) and commercialized animal feeds in Algeria. We highlight the potential toxic effects of the aflatoxigenic strains in the analyzed commodities. These results could help decision makers to set prevention and control strategies especially regarding low moisture content coupled with optimum storage conditions that are recommended to avoid triggering of aflatoxin production in Algerian animal feeds.

Key words: *Aspergillus* section *Flavi*, Polyphasic identification, Animal feeds, Aflatoxins, Algeria

Fusarium and *Aspergillus* spp. are classified by the Advisory Committee on Dangerous Pathogens as hazard group 2 biological agents under the Control of Substances Hazardous to Health Regulations 2002 (as amended) (Approved List of Biological Agents (http://www.hse.gov.uk/pubns/misc208.pdf) and as such can cause human disease and may be a hazard to employees; they are unlikely to spread to the community and there is usually effective prophylaxis or treatment available.

1 INTRODUCTION

In Algeria, animal feeds constituents include mainly maize followed by soybean, wheat bran and barley, with small amounts of limestone and a vitamin-mineral premix (USDA, 2018). In addition to the local production, Algerian authorities manage the importation of such commodities depending on local demand and international market prices. However, no information is available on the occurrence of aflatoxigenic fungal contamination (pre-harvest, post-harvest or during transportation) of the imported grains.

Animal feeds are subject to contamination by a wide range of fungi able to produce toxic secondary metabolites known as mycotoxins. Among these fungi, species belonging to the genus *Aspergillus* can produce aflatoxins (AFs), which present a serious health risk for both humans and animals. Aflatoxin B1 (AFB1) is considered as the most toxic aflatoxin and it is extremely implicated in humans hepatocellular carcinoma (IARC, 2002).

Many species of *Aspergillus* are widely known for their aflatoxins production (Perrone, Gallo, & Logrieco, 2014; Frisvad et al., 2019). Among the different species of the section *Flavi* the most predominant producers are: *Aspergillus flavus* producer of AFB1 and aflatoxin B2 (AFB2) and/or cyclopiazonic acid (CPA), and *A. parasiticus* that produce both aflatoxins B and G (AFB and AFG) but not CPA (Giorni, Magan, Pietri, Bertuzzi, & Battilani, 2007). Other strains of this section can also produce other mycotoxins such as *A. alliaceus* producer of ochratoxin A (OTA), largely found in soil samples and occasionally observed in food (e.g. figs and coffee) (Frisvad et al., 2007; Frisvad et al., 2019).

Aspergillus genus is well characterized by a combination of morphological (macro- and microscopic features), biochemical and molecular methods (Frisvad et al., 2019). The genus is complex and always evolving, with many overlapping morphological and biochemical characteristics between species or even with intraspecific polymorphism (Frisvad et al., 2019). Molecular methods applied for the identification of a large number of *Aspergillus* species were

historically based on the Internal Transcribed Spacer (ITS) region (White, Bruns, Lee, & Taylor, 1990) but are now expanding to include a set of other genes such as the calmodulin gene (CaM), beta-tubulin (BenA) and RNA polymerase II subunit (RPB2) (Carbone & Kohn, 1999; Glass & Donaldson, 1995; Samson et al., 2014; Okoth et al., 2018; Frisvad et al., 2019).

Based on the combinations of AFs and CPA production, Giorni et al., (2007) proposed seven chemotypes of *Aspergillus* section *Flavi*: (i) more production of AFB1 versus AFB2 and produce CPA (chemotype I); (ii) more production of AFB2 versus AFB1 and produce CPA (chemotype II); (iii) produce only AFB (chemotype III); (iv) produce only CPA (chemotype IV); (v) non-producers of AFB and CPA (chemotype V); (vi) produce all AFB, AFG and CPA (chemotype VI), and (vii) produce AFB and AFG (chemotype VII).

To the best of our knowledge, the incidence of *Aspergillus* section *Flavi* in animal feeds in Algeria is relatively unknown, although a few studies were conducted on wheat (Riba, Mokrane, Florence, Lebrihi & Sabaou, 2008; Riba et al., 2010; Zebiri et al., 2018), nuts (Riba, Matmoura, Mokrane, Mathieu, & Sabaou, 2013; Guezlane-Tebibel, Bouras, Mokrane, Benayad, & Mathieu, 2013), spices (Azzoune et al., 2016), and dried fruits (Ait Mimoune et al., 2018; Ait Mimoune, Riba, Verheecke, Mathieu, & Sabaou, 2016). Thus, the present investigation aimed to identify *Aspergillus* section *Flavi* species isolated from animal feeds and their corresponding raw materials (maize, soybean, wheat bran and barley) using a polyphasic approach which involved morphological, chemical and molecular methods, and to evaluate their distribution and toxigenicity potential. Considering the important consumption rate of animal feeds in Algeria and the need to ensure the safety of animals and humans, this work focuses to clearly evaluate the potential risk of aflatoxigenic *Aspergillus* population.

2 MATERIALS AND METHODS

2.1 Fungal isolates and culture conditions

The direct plating method described by Pitt & Hocking (2009) were applied to isolate fungi from the maize and barely samples. From each sample, a 100 g seeds was superficially disinfected by immersion in 0.5% sodium hypochlorite solution for 1 min, followed by 3 times washing with sterile distilled water. Then, the kernels were plated in Dichloran Rose Bengal Chloramphenicol medium (DRBC) (King, Hocking, & Pitt, 1979). Five plates containing 10 kernels were prepared for each sample, resulting to a total of 50 kernels per sample. The plates were incubated at 25°C for 5 days. For the remaining samples (soybean, wheat bran, poultry feed and cattle feed), the dilution plating technique was applied. Ten grams of each ground sample were suspended in 90 mL of sterile water to obtain a dilution of 10^{-1}. Serial decimal dilutions up to 10^{-4} were then prepared. A 0.1 mL aliquot of each dilution was inoculated on plates containing DRBC medium. All samples were processed in triplicate and plates were incubated at 25°C for 7 days (Pitt & Hocking, 2009).

One hundred and seventy-two isolates of *Aspergillus* section *Flavi* were isolated from 57 animal feeds collected from several Algerian regions between 2014 and 2015 (Supplementary Table S1). Seventy-six of these strains were isolated from maize (corn), 18 from soybean, 24 from wheat bran and six from barley intended for animal consumption, 39 from poultry feed and nine from cattle feed samples. All isolates were maintained in 30% glycerol at -20°C and grown on Potato Dextrose Agar (PDA) in the dark for seven days at 25°C whenever needed for further studies.

2.2 Morphological characterization

The morphological and physiological characteristics of all isolates were studied. The spore suspension of each isolate was obtained from a culture on PDA medium and incubated for seven days at 25°C. With a sterile platinum loop, the surface of the agar was scraped off slightly and then put into 2 mL Eppendorf tubes containing 1 mL of sterile agar solution (0.2% agar and 0.05% Tween 80 in 1 mL of distilled water) and stored at 4°C until use (Samson et al.,

2014). This suspension was used for three-points inoculation on 9 cm diameter Petri dishes containing 15 mL of standard identification media for *Aspergillus* species, namely Czapek Yeast Extract Agar (CYA), Malt Extract Agar (MEA), 25% Glycerol Nitrate Agar (G25N) and Yeast Extract Sucrose Agar (CY20S) (Samson et al., 2014). The Cultures were incubated for seven days, in the dark, at 25°C for the four media and also at 37°C and 42°C for CYA medium, and then assessed for colony color and diameter on the various media used, as well as presence and size of sclerotia.

To assay for sclerotia production, plates containing CYA medium were inoculated from a culture of 7 days. Cultures were incubated in the dark for 21 days at 30°C. Sclerotia were obtained by dispensing 10 mL of water with Tween 80 (0.01%) per plate and scraping the surface of culture plates (two replicate plates per isolate) over Whatman filter paper (N°1) and rinsing with tap water and finally air-dried (Novas & Cabral, 2002). Sclerotia formation was visualized, and their types were confirmed after measurement of their sizes under a photonic microscope (Motic).

2.3 Potential of mycotoxins production

2.3.1 Production and analysis of aflatoxins

For a preliminary screening of AFs production, strains were inoculated at a single central point on Petri dishes (Ø 6 cm) containing 10 mL of Coconut Agar Medium (CAM) (Davis, Iyer, & Diener, 1987) and incubated at 25°C for ten days in the dark. All the strains were observed for fluorescence under long-wave UV light (365 nm) after three and five days of incubation and scored as positive or negative. Then, three agar plugs were removed from each Petri dish, weighted, and 1 mL of methanol was added (Bragulat, Abarca, & Cabañes, 2001). After 1 h in methanol, the solution was centrifuged at 12,000 rpm for 10 min and filtered with a Q-Max PTFE hydrophilic filter (Ø 0.45 µm). After extraction, solutions of all the strains were spotted

on thin-layer chromatography (TLC) developed in chloroform/acetone 90:10: v/v system and observed under the UV light (365 nm).

AFs concentration of all extracts were measured by reversed phase HPLC Ultimate 3000 (Dionex, FR) coupled with a Coring Cell (Diagnostix Gmbh, GE) for post-column derivatization. The fluorescence detector (Ultimate 3000, RS Fluorescence Detector, Dionex) was fixed at an excitation wavelength λex = 365 nm and emission wavelength λem = 440 nm. A C18 Phenomenex Kinetex (5 µm, 250 x 4.6 mm) column was used. A 10 to 100 µL injection volume was used (depending on the level of AFs quantified) with a Dionex auto-injector. The mobile phase was methanol: acetonitrile (Fisher, UK):water (20:20:60) with 119 mg/L of potassium bromide (Acros Organics, BE) and 100 µL/L of 65% nitric acid (Merck, DA) were added. The flow rate was 1 mL/min. AFs quantification was done with standards (AFB1 produced by *A. flavus*, Sigma-Aldrich, FR) and the data were treated with Chromeleon software. AFs production was measured in ng/g of culture medium. The limit of detection (LOD) was 0.05 ng/g.

2.3.2 Cyclopiazonic acid detection

Since CPA production ability is a distinctive feature in section *Flavi*, the strains were tested for CPA in CYA medium. All strains were inoculated on 6 cm diameter plates and incubated at 25°C for 14 days, in the dark (Gqaleni, Smith, Lacey, & Gettinby, 1997). Then the methodology of Bragulat et al. (2001) was used for extraction. The obtained extracts were analyzed using thin layer chromatography (TLC). The detection of CPA was performed using ethyl acetate/propanol/ammonium hydroxide (40:30:20) as developing solvent system (Fernández Pinto, Patriarca, Locani, & Vaamonde, 2001). The plates were dipped first in a 2% solution of oxalic acid in methanol for two minutes. Subsequently, 20 µL of test samples and CPA standard were spotted on TLC plates. After plates development, CPA was visualized in daylight by treatment of the plates with Ehrlich's reagent (1 g of 4 dimethyl amino

benzaldehyde in 75 mL ethanol and 25 mL concentrated HCl) and appeared as a blue-purple spot. The LOD of the TLC technique was 1 µg/g.

2.3.3 OTA production

For OTA production, *A. alliaceus* was inoculated at a central point on a 6 cm Petri dish containing 10 mL of CYA medium (Bragulat et al., 2001) and incubated at 25°C for seven days in the dark. The strain culture was observed for fluorescence under long-wave UV light (365 nm) after 3 and 5 days and scored as positive (blue green fluorescence) or negative. Then, three agar plugs were removed and weighted, and 1 mL of methanol was added. After 1 h, the solution was centrifuged at 12,000 rpm for 10 min and filtered with a Q-Max PTFE hydrophilic filter (Ø 0.45 µm). The resulted solution was spotted on TLC and developed in toluene/ethyl acetate/90% formic acid (5:4:1, v/v/v). The OTA gave a blue green color after development and the LOD of this technic is 0.5 ng/g.

2.3.4 Aspergillic acid production

A. flavus and *parasiticus* agar (AFPA) medium was used in order to confirm the production of aspergillic acid. All isolates were cultured on this medium for 3 to 5 days at 25°C, in the dark. The appearance of orange color on the reverse side of the colony suggests the production of aspergillic acid (Pitt, Hocking, & Glenn, 1983).

2.4 Molecular characterization

2.4.1 DNA extraction

For molecular studies, among the 172 *Aspergillus* section *Flavi* isolates, 26 representative strains were molecularly characterized. The mycelium was incubated at 25°C, for five days in Potato Dextrose Broth (PDB) under shaking. Fungal DNA extraction was performed according to the method described by Liu, Coloe, Baird, & Pederson (2000). Concentration and purity of the DNA samples were measured with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

2.4.2 Polymerase Chain Reaction

For the phylogenetic analysis of the selected isolates, two regions of the genome were analysed, namely a part of CaM gene and the ITS region of the rRNA gene (including ITS regions 1 and 2 and the 5.8 S). DNA amplification was carried out for the ITS region using the primer sets ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The amplification of a portion of the CaM gene was accomplished with the primer pair CMD5 (5'-CCGAGTACAAGGARGCCTTC-3') and CMD6 (5'-CCGATRGAGGTCATRACGTGG-3') (Hong, Go, Shin, Frisvad, & Samson, 2005).

Amplification (for both CaM gene and ITS region) was set up following manufacturer's instructions in a total volume of 25 µL using a Taq Core kit 10 (MP Biomedicals, USA). The PCR reaction involved a denaturation cycle at 94°C for 4 min, followed by 30 cycles of a series of denaturation step at 94°C for 45 s, annealing for 45 s at 55°C for the ITS region and 57°C for the CaM gene, and extension at 72°C for 50 s, followed by a final extension step at 72°C for 10 min. PCR products were checked by electrophoresis on agarose gel after staining with GelRed™ Nucleic Acid Gel Stain (Biotium, USA). The size of the amplicons was estimated using a 1 kb DNA leader (Promega corporation, USA). PCR amplicons were sequenced by Genewiz (Beckman Coulter Genomics, UK) using the same primer sets used for amplification.

2.4.3 Phylogenetic analysis

For more accurate identification, each obtained sequence were subjected to BLAST search using a pairwise sequence alignment tool at Westerdijk Fungal Biodiversity Institute (http://www.westerdijkinstitute.nl/).

The obtained 26 calmodulin sequences were compared to 30 references sequences retrieved from GenBank, which corresponded to the most important strains currently identified in section *Flavi*. *Aspergillus niger* NRRL 326T (accession number: EF661154.1) was used as an outgroup. Concerning the ITS region, the obtained sequences (n=19) were compared to 24

type-strain sequences retrieved from GenBank. *Neopetromyces muricatus* CBS 112806 (accession number: FJ491585.1) was used as an outgroup. Alignment was made with MUSCLE (Edgar, 2004) in the MEGA version 7 software (Kumar, Stecher, & Tamura, 2016) than corrected and trimmed manually when needed. Thus, sequences of 540 bp and 550 bp were obtained for CaM gene and ITS region, respectively.

All the analyzed strains and their corresponding GenBank accession numbers for both CaM gene and ITS region are showed in Supplementary Table S2.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7 (Kumar et al., 2016). The evolutionary history was inferred by using the Maximum Likelihood method with 1,000 bootstraps replicates based on the Tamura-Nei model. The evolutionary distances were calculated using the Tamura-Nei method embedded in the MEGA package and are expressed in units of the number of base substitutions per site (Tamura & Nei, 1993). For the CaM gene the analysis involved 56 nucleotide sequences and there were a total of 512 positions in the final dataset. The analysis for the ITS region involved 43 nucleotide sequences, all positions with less than 95% site coverage were eliminated and there were a total of 548 positions in the final dataset.

3 RESULTS

3.1 Morphological characterization

Morphological characterization of the strains isolated from animal feeds and corresponding raw materials are summarized in Table 1 and Table 2. Four distinct morphotypes could be distinguished based on macro and microscopic characteristics: appearance on CYA medium, colony size and color, conidia morphology, vesicle seriation, absence or presence of sclerotia and their size.

The first morphotype was represented by 135 isolates of *A. flavus* (78.5%), which had yellow green colonies, smooth to finely rough spores with thin walls, and predominantly

biseriate. The second morphotype included three isolates (1.7%) of *A. parasiticus* with dark green colonies, rough spores with thick walls, and predominantly uniseriate. The third morphotype was represented by 33 isolates (19.2%) of *A. tamarii*, with dark brown color, rough spores, and predominantly uniseriate. The fourth morphotype included only one isolate of *A. alliaceus*, with white color, smooth spores, and biseriate. All the isolates were tested for their capacity to grow on CYA medium (25°C, 37°C and 42°C) and at 25°C only for CY20S, MEA, G25N. All cultured isolates on MEA and CY20S at 25°C and CYA at both 25 and 37°C showed colony diameters ranging from 32 to 58 mm after 7 days of incubation. The colony diameters on CYA at 42°C of all *A. flavus* and *A. parasiticus* isolates did not exceed 37 mm; while, *A. tamarii* and *A. alliaceus*, showed colony diameters less than 20 mm. Out of 172 *Aspergillus* section *Flavi* isolates, 30.8% were able to produce sclerotia.

3.2 Chemical characterization

Based on AFs and CPA production patterns, a total of 171 strains were classified into four chemotypes (Table 3), and only one strain of *A. alliaceus* produced OTA.

The 135 strains of *A. flavus* morphotype were classified into three chemotypes, among them, 114 (66.3%) produced AFB and CPA (chemotype I), 10 (5.8%) produced only CPA (chemotype IV) and 11 (6.4%) did not produce AFB, AFG and CPA under the tested conditions. After analyzing the color of the reverse side of the colony on AFPA medium, all these strains were positive for their ability to produce aspergillic acid, except one isolate which gave a cream color.

The 33 strains (19.2%) of *A. tamarii* had brown color on the reverse side of AFPA medium and produced only CPA without AFs while, *A. parasiticus* isolates (1.7%) had an orange color on reverse side of AFPA medium and produced both AFB and AFG but not CPA.

Isolates with the ability to produce AFB1, AFB2 and CPA (chemotype II), AFB only (chemotype III) and AFB, AFG and CPA (chemotype VI) were not detected in the present study.

Analysis of AFs production by fluorescence in TLC showed a good correlation with the HPLC results. For the fluorescence detection on CAM, only 5.1% (6 isolates) showed false-negative results. A total of 31.6% of the tested strains were not able to produce AFs, while 68.4% were able to produce AFs with different concentrations (Figure 1). Isolates of chemotypes IV and V were not able to produce any AFs, but a great variability was observed in chemotype I. In fact, the highest AFB1 production (>10,000 ng/g) was recorded in 44 isolates (25.7%) and the lowest production (<100 ng/g) was noted in 8 isolates (4.7%). Co-occurrence of AFs and CPA was found in 114 isolates of chemotype I, while the chemotype IV produced only CPA, and the chemotype VII produced only AFB and AFG with a concentration of AFB1 between 500 and 1000 ng/g.

3.3 Molecular characterization

A polyphasic approach was adopted for the identification of our 172 isolates. The combination of morphotype and chemotype results allowed us to subdivide our strains to 5 phenotypic groups (Table 3) in addition to another phenotype of *A. alliaceus*. From these groups, 26 and 19 representative strains were subjected to molecular analysis for a part of the CaM gene and the ITS region, respectively. For the CaM gene, among the 26 tested strains, 20 were phylogenetically related to *A. flavus* with a similarity ranging between 99.3% and 100%. Most of strains (16 out of 20) belonged to the chemotype I, the others having chemotype IV (3 strains) and V (one strain). In addition, two strains (chemotype VII) were related to *A. parasiticus* (99.65% similarity), 3 other strains (chemotype IV) were related to *A. tamarii* (100% similarity), and one strain was related to *A. alliaceus* with a similarity of 99.6%. The obtained results of the ITS region confirm those obtained with the CaM gene. The results from

phylogenetic analysis indicated that both CaM gene and ITS region had a good level of resolution and led to clades that matched the obtained phenotypic groups (Figure 2 and 3).

4 DISCUSSION

We report in the present study, the identification of 172 strains of *Aspergillus* section *Flavi* in animal feeds from Algeria. These strains were isolated and morphologically identified based on colony color, conidia morphology, sclerotia presence and size and conidial head seriation. Furthermore, these strains were chemically characterized. Based on this identification, *A. flavus* strains were the predominant (78.5%), followed by 19.2% of *A. tamarii*, 1.7% of *A. parasiticus* and 0.6% of *A. alliaceus.* The dominance of *A. flavus* have been reported in other commodities from Algeria such as wheat and wheat products (Riba et al., 2010), dried fruits (Ait Mimoune et al., 2016), peanuts (Guezlane-Tebibel et al., 2013) and spices (Azzoune et al., 2016). Similar results were shown in other African countries like Tunisia for cereals (Jedidi, Cruz, González-Jaén, & Said, 2017; Jedidi et al., 2018), and Tunisian vineyards (Melki Ben Fredj et al., 2007), Morocco for wheat grains (Hajjaji et al., 2006) and Nigeria for maize and marketed poultry feed (Atehnkeng et al., 2008; Ezekiel, Atehnkeng, Odebode, & Bandyopadhyay, 2014).

All sclerotia produced by the *Aspergillus flavus* isolates were dark, hard with nearly spherical shape (globose to subglobose), and were larger than 400 µm (L strains). Many studies reported the dominance of the L strains from Algeria (Ait Mimoune et al., 2016; Guezlane-Tebibel et al., 2013; Riba et al., 2010), Nigeria (Atehnkeng et al., 2008; Ezekiel et al., 2013; Ezekiel, Atehnkeng, Odebode, & Bandyopadhyay, 2014), Italy (Giorni et al., 2007), Portugal (Rodrigues, Santos, Venâncio, & Lima, 2011; Rodrigues, Venâncio, Kozakiewicz, & Lima, 2009), Argentina (Astoreca, Dalcero, Fernández Pinto, & Vaamonde, 2011), Brazil (Baquião et al., 2013) and several Sub-Saharan countries in Africa (Probst, Bandyopadhyay, & Cotty, 2014).While others reported that S strain isolates were frequently found in high-temperature regions with relatively low rainfall like in Eastern Kenya (Probst, Bandyopadhyay, Price, &

Cotty, 2011; Probst, Schulthess, & Cotty, 2010; Okoth et al., 2012) and North America (Cardwell & Cotty, 2002).

Four chemotypes were distinguished based on the results of AFs and CPA production. More than half of the isolates belonged to the chemotype I (66.3%). These results are in agreement with those of Astoreca, Dalcero, Fernández Pinto, & Vaamonde (2011), Azzoune et al., (2016) and Giorni et al., (2007), which showed that the group producing both AFB and CPA was the most dominant. Our results also showed that *A. flavus* strains were more variable in their mycotoxigenic potential as it was reported in other studies (Razzaghi-Abyaneh et al., 2006; Rodrigues et al., 2011; Rodrigues et al., 2009; Vaamonde, Patriarca, Fernández Pinto, Comerio, & Degrossi, 2003).

Furthermore, *A. parasiticus* showed a homogenous group of chemotype VII, which produced AFB and AFG but no CPA, while *A. tamarii* group showed only one chemotype (IV). In this study, chemotypes II (produce more AFB2 than AFB1), chemotype III (producers of AFB but not CPA) and chemotype VI (producers of AFB, AFG and CPA) were not found. The same results were reported in Algeria by Ait Mimoune et al. (2016) and Riba et al. (2010). The incidence of non-aflatoxigenic isolates representing chemotype V that was found in our study is relatively low (6.4%) in comparison with results reported by other authors (Razzaghi-Abyaneh et al., 2006; Rodrigues et al., 2009; Sánchez-Hervás, Gil, Bisbal, Ramón, & Martínez-Culebras, 2008; Vaamonde et al., 2003). Only one strain of *A. alliaceus*, characterised by white color, large and dark sclerotia with the ability to produce OTA but never AFs (Frisvad et al., 2019) was isolated.

Some of our strains could not be morphologically differentiated, which may be due to interspecific similarities and intraspecific variability (Rodrigues et al., 2011; Rodrigues et al., 2009). Thus, the adopted polyphasic approach based on morphological studies, mycotoxigenic

patterns and molecular analysis allowed us to identify our isolates to species level (Rodrigues et al., 2009; Samson & Varga, 2009; Samson et al., 2014).

Results from the molecular analysis were generally in agreement with those from morphological and chemical studies. Based on the phylogenetic tree, the selected strains were divided into major clades regarding the CaM gene and ITS region namely, *A. flavus*, *A. tamarii* and *A. alliaceus*. In the first *A. flavus* clade, two sub-clades are formed; *A. flavus* represented by three chemotypes (chemotypes I and IV that are aflatoxigenic and chemotype V that include non aflatoxigenic strains) and *A. parasiticus* which is represented by only one chemotype (VII). All strains of *A. flavus* sub-clade were closely related to *A. flavus* and *A. oryzae* species independent of their aflatoxigenic ability. Several authors reported that *A. flavus* and *A. oryzae* are almost genetically identical highlighting the problem of species identification (Geiser, Pitt, & Taylor, 1998; Rokas et al., 2007). The cream color of the reverse side of the strain BF1 colony on AFPA could lead to its classification as *A. oryzae* species (Frisvad et al., 2019; Rodrigues et al., 2009). Also, for the second sub-clade, the two strains BP17 and BP31 were related to *A. parasiticus* and *A. sojae*. Frisvad et al. (2019) stated that *A. sojae* is the domesticated form of *A. parasiticus.* These two species are morphologically and chemically very similar. *A. sojae* does not produce aflatoxins while *A. parasiticus* is known to have the ability to produce AFB and AFG but not CPA.

Concerning the *A. tamarii* clade, only one chemotype (represented by the isolates BT29, BT30 and BT32) was identified. In addition, the last clade contains only one strain that was affiliated to *A. alliaceus* and can produce OTA. Similar results were found by Frisvad et al. (2019) who reported that most isolates of *A. alliaceus* strains produced large amounts of OTA.

In conclusion, we evaluated the occurrence of *Aspergillus* section *Flavi* isolated from raw materials (maize, soybean, wheat bran and barley) and commercialized products. In our knowledge, this is the first report of the identification and characterization of *Aspergillus*

section *Flavi* to the molecular level in animal feeds in Algeria. A high frequency of AFs producers was revealed, amplifying the need to assess aflatoxins contamination in Algeria. Furthermore, an important proportion of isolates were capable of producing CPA indicating potential additional risk. Since aflatoxins production is also related to temperature and relative humidity, decision makers must take these parameters into consideration when establishing prevention and control strategies for storage conditions of Algerian animal feeds.

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CONFLICT OF INTEREST

There is no conflict of interest.

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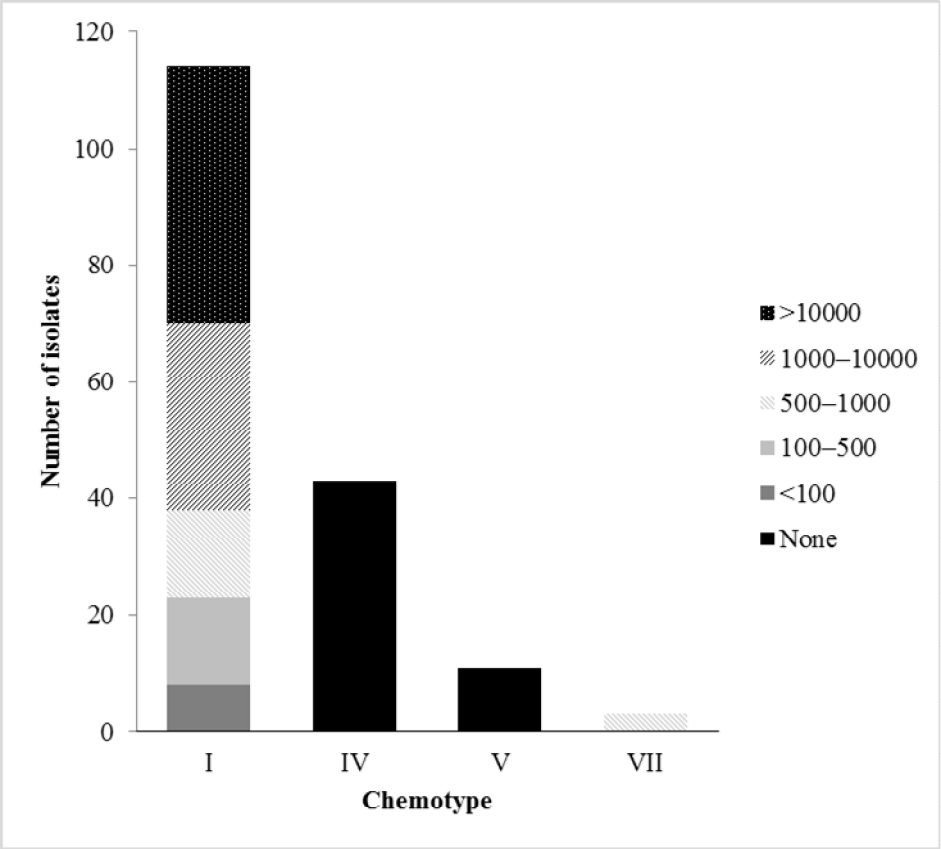

FIGURE 1 Production of AFB1 (ng/g) by *Aspergillus* section *Flavi* strains depending on their chemotype.

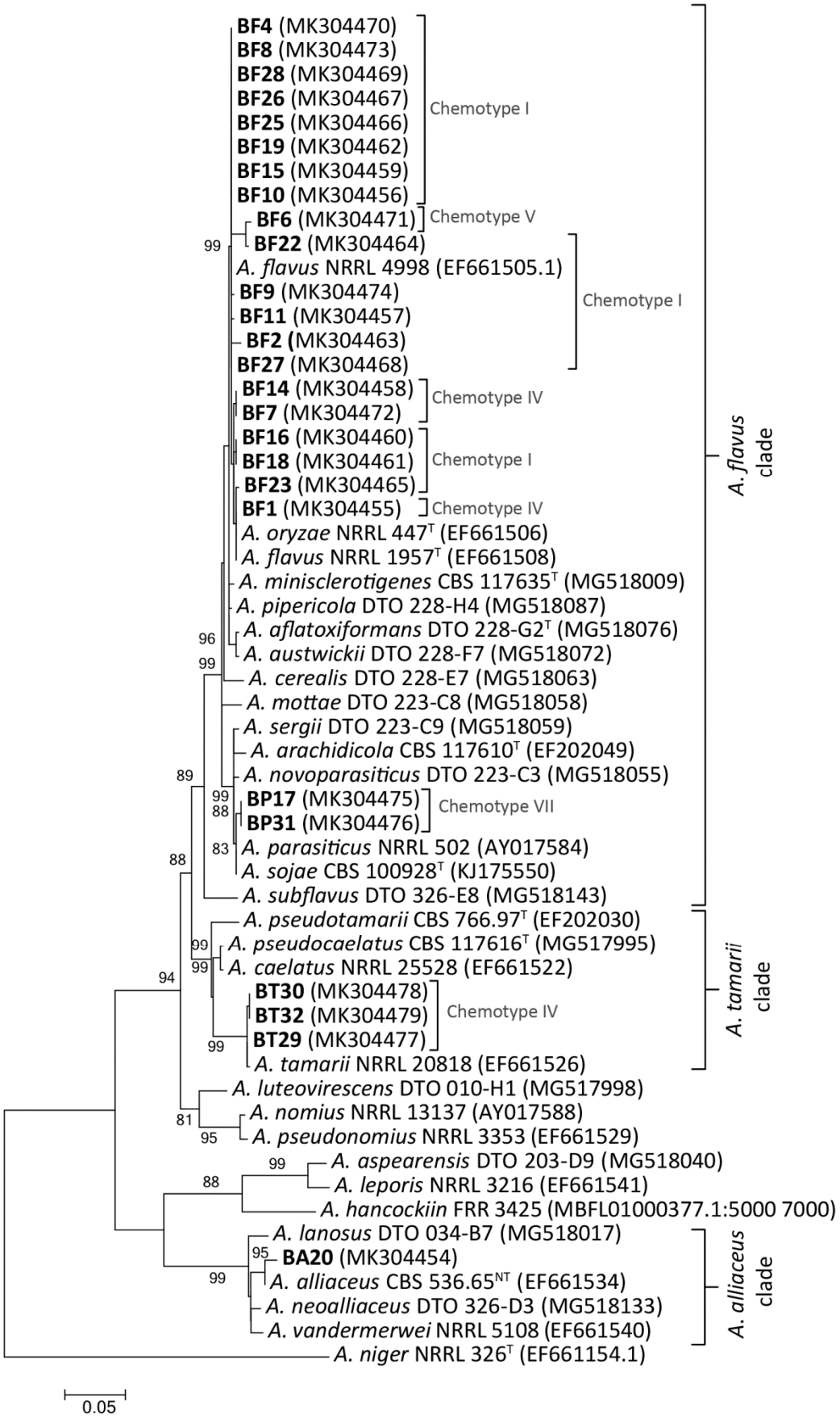

FIGURE 2 Phylogenetic tree for a part of CaM gene. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Numbers above branches are bootstrap values. Only values above 75% are indicated.Bar, 0.05 nucleotide substitutions per site.

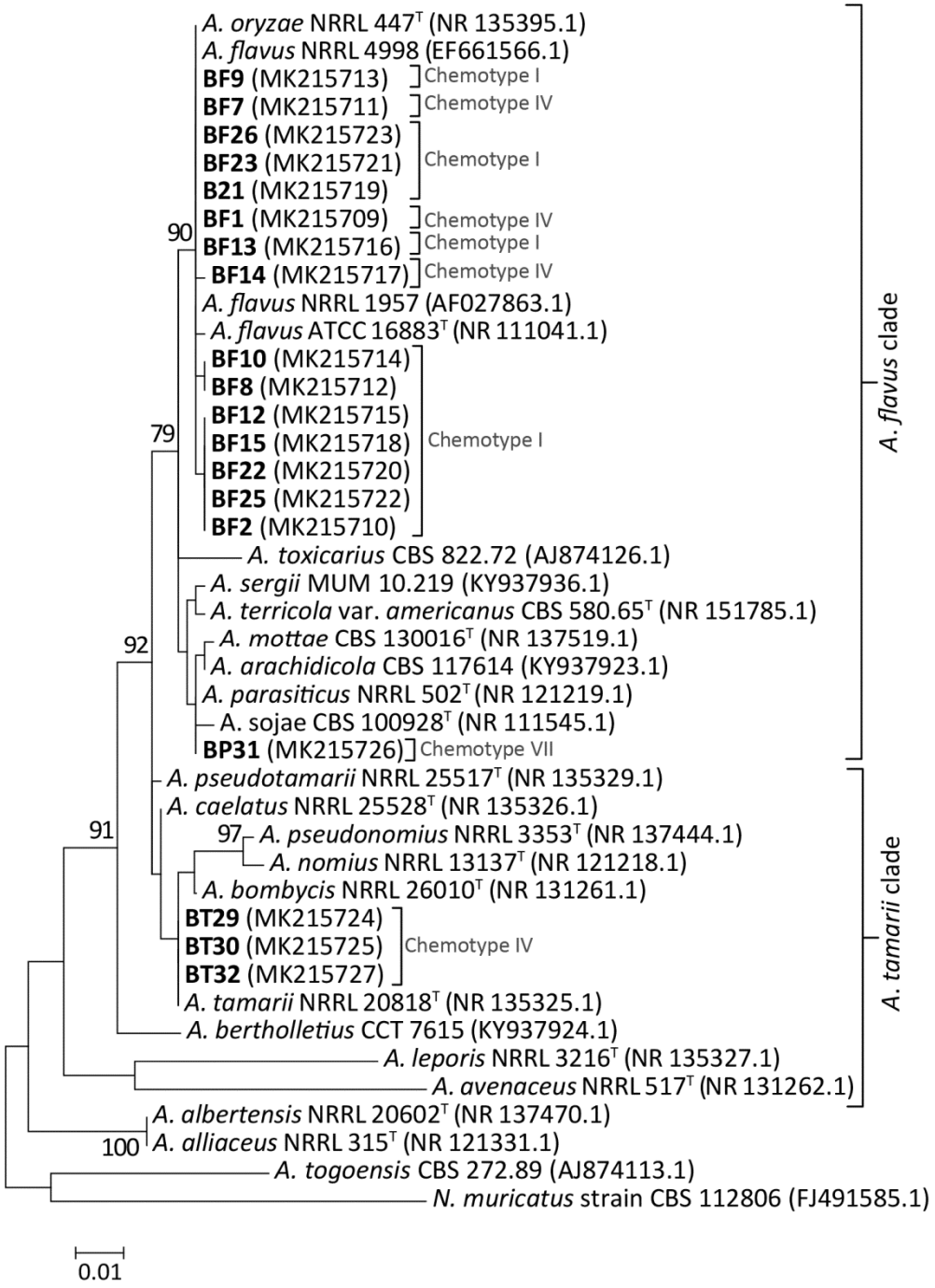

FIGURE 3 Phylogenetic tree for ITS region. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Numbers above branches are bootstrap values. Only values above 75% are indicated. Bar, 0.01 nucleotide substitutions per site.

TABLE 1 Morphological characterization of *Aspergillus* section *Flavi* isolates

Morphotype	Number of isolates	Phenotypic features			
		Sclerotia size (µm)[a]	Seriation[b]	Conidia	Colony color
A. flavus	39	400-800	b or b/u	Smooth; finely rough	Yellow-green
	75	-	b or b/u	Smooth; finely rough	Yellow-green
	6	400-1000	b or b/u	Smooth	Yellow-green
	4	-	b or b/u	Smooth	Yellow-green
	7	500-800	b or b/u	Smooth	Yellow-green
	4	-	b or b/u	Smooth	Yellow-green
A. parasiticus	3	-	u	Rough	Dark-green
A. tamarii	33	-	u/b	Rough	Dark-brown
A. alliaceus	1	500-1000	b	Smooth	White

a: −: no sclerotia observed, u: uniseriate; b: biseriate; b/u: predominantly biseriate; u/b: predominantly uniseriate.

TABLE 2 Colony diameter of *Aspergillus* section *Flavi* species isolated in this study

Morphotype	Chemotype	Colony diameter (mm)[a]					
		CYA 25°C	**CYA 37°C**	**CYA 42°C**	**MEA 25°C**	**G25N 25°C**	**CY20S 25°C**
A. flavus	I	37-53	35-56	20-35	32-54	27-40	42-57
	IV	41-53	35-54	31-37	39-55	30-40	53-58
	V	36-52	33-54	25-35	33-51	28-39	45-57
A. tamarii	IV	39-46	48-53	18-20	43-48	33-36	53-54
A. parasiticus	VII	37-52	45-57	21-34	39-54	34-41	55-57
A. alliaceus	/	54	43	12	45	41	56

a: range of the average of 3 colonies per strain in mm after 7 days of incubation on different media.

TABLE 3 Chemical characterization of *Aspergillus* section *Flavi* isolates

Chemo-types	Number and percentage of isolates	Toxigenicity						Possible identification
		AFB1	AFB2	AFG1	AFG2	CPA	Reverse on AFPA	
I	114 (66.27%)	++/+	+	-	-	+	Orange	*A. flavus*
IV	10 (5.8%)	-	-	-	-	+	Orange (9) and cream (1)	*A. flavus*
	33 (19.19%)	-	-	-	-	+	Brown	*A. tamarii*
V	11 (6.4%)	-	-	-	-	-	Orange	*A. flavus*
VII	3 (1.74%)	+	+/−	++	+	-	Orange	*A. parasiticus*

++ high intensity signal; +: medium intensity signal; +/−: low signal; −: not detected